Ion channel mechanisms of rat tail artery contraction-relaxation by menthol involving, respectively, TRPM8 activation and L-type Ca^{2+} channel inhibition

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Running title: Vascular tone and TRPM8 agonists

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Key words: TRPM8, TRPM8 agonists, voltage-gated Ca^{2+} channels, vasodilatation, vasoconstriction

Summary

Transient receptor potential melastatin 8 (TRPM8) is the principal cold and menthol receptor channel. Characterized primarily for its cold sensing role in sensory neurons, it is expressed and functional in several non-neuronal tissues, including vasculature. We previously demonstrated that menthol causes variable mechanical responses (vasoconstriction, vasodilatation or biphasic reactions) in isolated arteries, depending on vascular tone. Here we aimed to dissect the specific ion channel mechanisms and corresponding Ca^{2+} signalling pathways underlying such complex responses to menthol and other TRPM8 ligands in rat tail artery myocytes using patch-clamp electrophysiology, confocal Ca^{2+} imaging and ratiometric Ca^{2+} recording. Menthol (300 μM, a concentration typically used to induce TRPM8 currents) strongly inhibited L-type Ca^{2+} channel current (L-I_{Ca}) in isolated myocytes, especially it's sustained component, most relevant for depolarisation-induced vasoconstriction. In contraction studies, with nifedipine present (10 μM) to abolish L-I_{Ca} contribution to phenylephrine (PE)-induced vasoconstrictions of vascular rings, a marked increase in tone was observed with menthol, similarly to resting (i.e. without α-adrenoceptor stimulation by PE) conditions, when L-type channels were mostly deactivated. Menthol-induced increases in PE-induced vasoconstrictions could it be inhibited both by the TRPM8 antagonist AMTB (thus confirming specific role of TRPM8), and by cyclopiazonic acid treatment to deplete Ca^{2+} stores, pointing to a major contribution of Ca^{2+}-release from the sarcoplasmic reticulum in these contractile responses. Immunocytochemical analysis has indeed revealed co-localisation of TRPM8 and $InsP_3$ receptors . Moreover, menthol Ca^{2+} responses, which were somewhat reduced under Ca^{2+}-free conditions, were strongly reduced by cyclopiazonic acid treatment to deplete Ca^{2+} store, while caffeine-induced Ca^{2+} responses were blunted in the presence of menthol. Finally, two other common TRPM8 agonists, WS-12 and icilin, also inhibited L-I_{Ca}. With respect to L-I_{Ca} inhibition, WS-12 is the most selective agonist. It augmented PE-induced contractions, while any secondary phase of vasorelaxation (as with menthol) was completely lacking. Thus, TRPM8 channels are functionally active in rat tail artery myocytes and play a distinct direct stimulatory role in control of vascular tone. However, indirect effects of TRPM8 agonists, which are unrelated to TRPM8, are mediated by inhibition of L-type Ca^{2+} channels, and largely obscure TRPM8-mediated vasoconstriction. These findings will promote our understanding of

vascular TRPM8 role, especially the well known hypotensive effect of menthol, and may also have certain translational implications (e.g. in cardiovascular surgery, organ storage and transplantation, Raynaud's phenomenon).

New & Noteworthy

Although the cold and menthol receptor, calcium-permeable TRPM8 channel is expressed in vascular smooth muscles, surprisingly menthol causes vasorelaxation. Here we dissect the true contractile response of rat tail artery myocytes to TRPM8 activation, which is normally "masked" by the nonspecific inhibition of voltage-activated L-type calcium channels resulting in vasorelaxation.

Key words: TRPM8 agonists, voltage-gated Ca^{2+} channels, vasodilatation, vasoconstriction

Abbreviations list

AMTB, N-(3-aminopropyl)-2-{[(3-methylphenyl)methyl]oxy}-N-(2-thienylmethyl)-benzamide hydrochloride salt; L-I_{Ca}, Ca^{2+} current via L-type Ca^{2+} channels; L-VGCC, L-type voltage-gated Ca^{2+} channel; PE, phenylephrine; PSS, physiological salt solution; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TRP, transient receptor potential; TRPM, transient receptor potential melastatin; VGCC, voltage-gated Ca^{2+} channel; VSM, vascular smooth muscle; VSMC, vascular smooth muscle cell.

Introduction

Transient receptor potential melastatin member 8 (TRPM8) is an cation channel best known for its role in sensory nerve endings where it is activated by cold temperatures and cooling compounds, such as menthol and icilin (23, 25, 27). In nerve terminals, TRPM8 channel opening initiates membrane depolarization accompanied by an acceleration of action potential discharge that ultimately result in the perception of environmental cold (3, 23). Although the role of TRPM8 is now well established as the primary sensor of thermal stimuli in the innocuous cold range (3, 12, 13), with a temperature threshold of ~26°C (25), the channel was first cloned and identified in prostate epithelia and several types of carcinoma (38), tissues in which temperature would not be expected to deviate substantially from that of the core body temperature. TRPM8 expression has now been found in several non-neuronal tissues including the bladder and male genital tract (34), lung epithelia (32), and in the vasculature (16, 17, 26, 35, 42, 43). Additionally, numerous endogenous molecules have been proposed to increase (phosphatidylinositol 4,5-biphosphate and lysophospholipids; 1, 2, 20, 30) and decrease (arachidonic acid; 2, 5) TRPM8 activity and enzymes protein kinase A and protein kinase C have been proposed to regulate TRPM8 by altering its phosphorylated state (4, 5, 30), thus providing putative endogenous biochemical modulatory pathways for TRPM8, distinct from thermal stimuli. These findings raise important questions as to the whole spectrum of expression and functions of this truly polymodal protein that forms Ca^{2+}-permeable cation channels.

There is strong evidence to indicate TRPM8 gene expression in rat and mouse vascular smooth muscle (VSM; 17, 35, 42, 43). As a Ca^{2+}-permeable cation channel (P_{Ca}/P_{Na}~1-3; 25, 27), activation of TRPM8 located on the plasma membrane and/or the membrane of the sarcoplasmic reticulum (SR) of these VSM cells (VSMC) would be expected to induce increased intracellular Ca^{2+} ($[Ca^{2+}]_i$). In a study by Yang *et al*. (43), the application of menthol (300 μM) to transiently cultured rat aortic and pulmonary VSMCs resulted in the expected robust increases in $[Ca^{2+}]_i$ that were dependent on the presence of extracellular Ca^{2+} and could be blocked by the non-selective cation channel inhibitor Ni^{2+} (300 μM), while the L-type voltage-gated Ca^{2+} channel (L-VGCC) blocker nifedipine (1 μM) had no effect (43). These findings indicated that menthol-induced responses in rat aortic and pulmonary

VSMCs were entirely mediated by Ca^{2+} influx, suggesting TRPM8 expression on the plasma membrane of these cells.

In our earlier work we used an organ bath tensiometric preparation along with TRPM8 pharmacological modulators, menthol and icilin, as well as KCl and the α_1-adrenoceptor agonist, phenylephrine (PE), in order to observe the consequences of putative TRPM8 activation on the tone of vascular rings, from several major arteries and during different contractile states (17). From these functional experiments, we observed complex effects of TRPM8 agonists on vascular contractility, causing prominent vasodilatations when applied during PE- and high-K^+-induced vasoconstrictions, while menthol (300 µM) consistently caused vasoconstrictions when applied to rat tail artery vascular rings at rest. These are disparate effects as menthol-induced activation of TRPM8 would be expected to invariably induce VSM contraction by its virtue of raising $[Ca^{2+}]i$ (43). To add to the puzzle, systemic and chronic dietary administration of menthol is known to have a pronounced hypotensive effect (30, 35), and in our earlier study we also observed vasodilation of cutaneous blood vessels *in vivo* after topical menthol application (17). Such observations strongly imply that there may be other targets for menthol action on blood vessels, apart from TRPM8, for which it is presumed to be a selective agonist that can only be expected to cause Ca^{2+} mobilisation and vasoconstriction.

Menthol-induced vasorelaxation has been corroborated in a more recent study that systematically investigated this effect in different rat vascular beds (aortae, mesenteric and coronary arteries) and confirmed its wide-spread existence throughout the vascular system. In this study, Cheang *et al*. (10) hypothesised that such effect could be explained by the dominant menthol inhibition of VGCC, activation of which normally initiates and maintains VSM contraction during membrane depolarisation, and provided Ca^{2+}-imaging and pharmacological evidence for this hypothesis. In this context, it should be noted that (i) smooth muscle and cardiac VGCC are molecularly similar (the principal transmembrane subunit α1 is $Ca_V1.2$ type; (9), and (ii) there is direct patch-clamp evidence for menthol-induced inhibition of cardiac L-type VGCC that occurs at concentrations which normally activate TRPM8 (IC_{50}=75 µM; (6). However, the hypothesis does not address the entire complexity of menthol action on vascular smooth muscles, as it contradicts measurements of $[Ca^{2+}]_i$ rises and vascular contractions of non-stimulated VSM and blood vessels induced by TRPM8 agonists (17, 43). Moreover, even in pre-

constricted vascular rings menthol induces notable initial contraction preceding relaxation (17). Thus, in the present study, our aims were (i) to identify the effects of TRPM8 agonists (mainly menthol as the most widely used one, and especially interesting as a common ingredient in many medicines and consumer products), on vascular tissues using a range of physiological and biophysical techniques and a much more rigorous set of experiments than previously used aiming to dissect the specific TRPM8-mediated effects, (ii) to further test the hypothesis that TRPM8 is functional in rat tail artery VSM but that the consequences of TRPM8 activation may be masked by non-specific effects of TRPM8 agonists, most likely on VGCC, and (iii) to directly test this latter possibility by patch-clamp measurements of L-type Ca^{2+} channel currents.

Some of the results have been communicated in an abstract form (24).

Methods

Ethical approval - All experimental procedures involving animals were in accordance with UK Animal Scientific Procedures Act (1986) and were approved by the Queen's University and University of Liverpool Animal Welfare and Ethics Committees.

Animal tissue - Experiments were performed on vessels freshly dissected from 8- to 12-wk-old male Sprague-Dawley rats. Tail arteries were removed from the whole length of the tail. The proximal 2–3 cm of the artery was taken for tensiometric studies, and the rest was used for smooth muscle isolation followed by Ca^{2+} measurement or patch clamp analysis.

Vascular smooth muscle isolation - Excised tail arteries were transferred to Ca^{2+}-free physiological salt solution (PSS) containing the following (in mM): 120 NaCl, 6 KCl, 1.2 $MgCl_2$, 10 HEPES, and 12 glucose, pH 7.4 (adjusted with NaOH). The artery was cleaned free of connective tissue and longitudinally cut. The endothelium was removed by gently rubbing of the luminal surface with fine stainless steel wire. The tissue was then sectioned into 5-mm lengths before being transferred to dissociation medium (DM) containing the following (in mM): 110 NaCl, 5 KCl, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10 Na_2HCO_3, 10 HEPES, 10 taurine, 0.5 EDTA, 10 glucose, 2 $MgCl_2$, and 0.16 $CaCl_2$, pH 7.4 (adjusted with NaOH). The tissue was digested at 37°C for 20 min in DM containing collagenase (type XI; 1 mg/ml), papain (1 mg/ml), BSA (0.4 mg/ml), and dithiothreitol (0.8 mM) and was then washed with Ca^{2+}-free PSS to stop digestion. Single myocytes were dispersed by trituration with a small-bore glass pipette.

Electrophysiology - Isolated vascular myocytes were plated in a CoverWell perfusion chamber (Grace Bio-Labs, Sigma-Aldrich Ltd, Dorset, UK) pressed-to-seal to glass coverslips and placed on the stage of an inverting microscope (Nikon Eclipse TE2000-S, Nikon Corp., Tokyo, Japan). 10 minutes prior to commencement of experiments, the cells were superfused with a modified PSS containing (in mM): NaCl 120, CsCl 6, HEPES 10, $MgCl_2$ 1.2, glucose 12, $CaCl_2$ 5, pH 7.4 at room temperature.

Borosilicate glass pipettes (Harvard Apparatus Ltd., UK) were fabricated and fire-polished to resistances of 3-4 MΩ and were back-filled with pipette solution

containing (in mM): 80 CsCl, 2 $MgCl_2$, 11 EGTA, 7 Na_2ATP, 0.5 Na_2GTP, 10 HEPES, and 5 creatine (pH 7.3 adjusted with CsOH). Pipette electrodes were mounted on a CV203BU head-stage connected to a patch clamp amplifier (Axopatch 200B, Molecular Devices, Union City, CA, USA). Whole-cell currents were filtered at 2 kHz (8-pole low-pass Bessel) and sampled at 5 kHz.

To determine the presence of a persistent Ca^{2+} current (L-I_{Ca}) through VGCCs (i.e. the so-called non-inactivating "window current" that occurs in specific range of potentials as an important determinant of vascular tone) in the tail artery VSMCs, a double-pulse voltage protocol was employed with a constant depolarising test potential to 10 mV, preceded by a variable amplitude prepulse ranging from -100 to 40 mV with a 10 mV increment (see *upper panel* of figure 1A).

A custom-made solution delivery system consisting of 8 pressurized reservoirs attached to an 8 channel solution delivery pen (AutoMate Scientific, Berkeley, CA, USA) with a single outlet needle (250 µm internal diameter; AutoMate Scientific) was used for fast solution application. The needle outlet was positioned in close proximity (0.1-0.5 mm) to the recorded cell and was used to superfuse the cell with the test compound ("bath solution", "vehicle" or "drug") until a steady-state response had been attained. In addition, a gravity-driven solution exchange attached to one side of the bath and vacuum to the other ensured fast and complete removal of test compounds from the perfusion chamber.

To investigate the direct effects of TRPM8 agonists on calcium currents, L-I_{Ca} was activated by applying 400 ms depolarizing pulses to a test potential of 10 mV from a holding potential of -60 mV at 10 s intervals. L-I_{Ca} parameters were measured as the peak inward current (L-$I_{Ca\text{-}Peak}$) achieved soon after the start of the depolarizing pulse and as the late non-inactivating current (L-$I_{Ca\text{-}Late}$) measured as the mean current during the final 10 ms of the 400 ms pulse. To allow quantitative comparison of experimental conditions, all currents were normalised such that L-$I_{Ca\text{-}Late}$ measured following 30 seconds incubation in nifedipine (10 µM) was set as baseline current for each cell studied.

Ratiometric Ca^{2+} recording – Isolated cells in low-Ca^{2+} PSS were loaded with Fura-2-AM (final concentration: 5 µM; Molecular Probes Inc, Invitrogen) and pluronic acid (final concentration: 2.5 mg/ml; Sigma-Aldrich) dissolved in DMSO for 25-30 minutes in a glass-bottomed recording chamber. Cells were washed with low-Ca^{2+} PSS after

the loading period for up to an hour at room temperature to allow de-esterification of the dye. A gravity-driven continuous flow of PSS ($[Ca^{2+}]$ = 2 mM) was used to superfuse the preparation for 10 minutes prior to experiment commencement, with excess solution removed by a suction line.

A photometric system consisting of an inverted microscope (Olympus IX50, Olympus UK Ltd., London, UK) and a Cairn monochromator (Cairn Research Ltd., Faversham, UK) with dual excitation (340 and 380 nm) were used to study $[Ca^{2+}]_i$ responses and their temporal characteristics in rat tail artery VSMCs upon the application of various pharmacological agents. Signals were recorded using a ×60 oil immersion objective (N.A. 1.3) and emitted fluorescence was measured at 510 nm from the side port of the microscope, via an adjustable rectangular window that allowed isolation of a region of interest around a selected cell. Acquisition software (pClamp 9, Molecular Devices Inc., CA, USA) was used for analysis and on-line computation of the F_{340}/F_{380} ratio.

Laser confocal Ca^{2+} imaging – similarly to Fura-2, the AM ester derivative of Fluo-3 (Fluo-3 AM, Invitrogen), with the addition of pluronic acid, was used to monitor Ca^{2+} signals in isolated myocytes using a laser-scanning confocal microscope (MR-A1, Bio-Rad laboratories, CA, USA) coupled to an inverted microscope (Nikon Eclipse TE300, Nikon Corp., Tokyo, Japan). On this system images were collected at one frame per second via oil-immersion objectives (×40, N.A. 1.4; and ×60, N.A. 1.4). The Fluo-3 dye was excited by an argon laser at 488 nm and emitted light was filtered through a 530-560 nm band-pass filter and detected using a photomultiplier tube. Data acquisition was controlled with Lasersharp software (Biorad, CA, USA) and analysis was performed using EZ-C1 FreeViewer software (Nikon Corp., Tokyo, Japan). In addition, fast Ca^{2+} imaging was performed using an Ultraview LCI spinning Nipkow disc, widefield Olympus IX70 inverted microscope (Perkin-Elmer Confocal Microscope System, Cambridge, UK), with a high sensitivity, Orca-ER cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) to capture emitted fluorescence. Images were collected at a rate of 33 frames per second via a ×60 water immersion objective (NA 1.2) with fluo-4 loaded cells excited by an argon laser at 488 nm and emitted fluorescence measured at 510 nm. Data acquisition and analysis were performed using Perkin-Elmer Microscope System software.

Contraction studies – Tail artery sections with endothelium removed had mechanical responses recorded as previously described (17). The condition of the tissue was initially tested by adding KCl (60 mM) and rejecting tissue that did not respond with a robust contraction (>0.5 g tension). One of three basic protocols was then conducted. 1) Vessels were examined for a contractile response to menthol alone (0.1-1 mM; experiment performed at room temperature). 2) Vessels were contracted with PE (2 µM) and menthol (300 µM) was added during contraction (having allowed 10 min for contraction to stabilize). 3) To eliminate the involvement of L-type VGCC in TRPM8 agonist-induced responses vessels were incubated with the L-type VGCC antagonist nifedipine (10 µM) for 10 minutes prior to repeating the second protocol. Vascular responses to TRPM8 agonist application were measured as the peak constriction induced (peak response) and the tone remaining 10 minutes after application (late response) relative to PE-induced tone immediately prior, as illustrated in Fig. 4B. Experiments examining the effects of WS-12 (50 µM) were performed and data analysed in the same manner. All summary data presented have been normalized to the PE-vasoconstriction amplitude 10 minutes after PE application.

To determine the specificity of the menthol-induced responses, tail artery vascular rings were preincubated with the TRPM8 antagonist N-(3-aminopropyl)-2-{[(3-methylphenyl)methyl]oxy}-N-(2-thienylmethyl)-benzamide hydrochloride salt (AMTB, 10 µM), in addition to nifedipine, for 10 minutes before repeating protocol 3.

Immunocytochemistry – Isolated tail artery VSMCs were fixed in 2 % paraformaldehyde (PFA; Sigma-Aldrich) for 4 minutes, washed for 1-1½ hours in phosphate buffered saline (PBS), changing solution every 15 minutes, and blocked in PBS containing 1 % BSA and 0.05 % triton-X (Invitrogen) for 1 hour (all at room temperature). To visualise TRPM8 subcellular localisation, cells were incubated with rabbit anti-TRPM8 primary (ACC-049; Alomone Labs, Jerusalem, Israel; dilution of 1:200) overnight at 4 °C. Cells were again washed in PBS for 1-1½ hours, before being incubated for 1 hour with Alexa Fluor 488 donkey anti-rabbit IgG (A21206; Invitrogen Molecular Probes; dilution of 1:200) in the dark. After a further washing, slides were completed by applying Vectashield hard-setting mounting medium (Vector labs Inc.) containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) to counterstain the cell nuclei, and a glass coverslip. Slides were examined using

confocal microscopy. In addition, the anti-TRPM8 antibodies were used in combination with goat primary antibodies for type I $InsP_3$ receptor ($InsP_3R1$) (sc-6093; Santa Cruz Biotechnology Inc., CA, USA; dilution of 1:100) and secondary Alexa Fluor 594 chiken anti-goat IgG (A21468; Invitrogen Molecular Probes; dilution of 1:200) to determine if co-localisation of TRPM8 and $InsP_3R1$ was evident. 488 nm (argon) laser was used to view Alexa 488 staining and 561 nm (HeNe) laser - Alexa 594 staining. Control experiments were performed by the omission of primary antibodies to determine the specificity of the secondary antibody or by the omission of both primary and secondary antibodies to detect auto-fluorescence in the cells of interest.

Statistical analysis - Data are expressed as mean ± standard error of the mean (SEM) with N for the number of animals and n for the number of samples. Statistical significance of differences between control and experimental treatments were determined using Student's paired or unpaired *t*-tests as appropriate or one-way ANOVA for multiple comparisons. In contractile experiments, values for each condition, i.e. PE after 10 minutes, menthol peak and after 10 minutes (late) were compared by one-way ANOVA for repeated measures followed by Tukey's post hoc test. Peak and late values in control conditions and in the presence of nifedipine were compared by Student's t-*t*est. *P* values of <0.05 were accepted as statistically significant, denoted in the graphs by *; $P<0.01$ and $P<0.001$ are denoted as ** and ***, respectively.

Drugs - All drugs were supplied by Sigma-Aldrich (Dorset, UK), except for AMTB (Tocris Bioscience, Abingdon, UK).

Results

I_{Ca} in rat tail artery VSMCs and the effects of TRPM8 agonists

To investigate parameters of a persistent L-I_{Ca} in tail artery VSMCs, eight cells were recorded using the double-pulse protocol (mean membrane capacitance C_m=42.2±4.0 pF; N=4) and activation (**m**∞) and inactivation (**h**∞) curves were constructed from peak amplitudes of L-I_{Ca} during the pre-pulse and test steps in a conventional manner (Fig. 1A,B). The potentials of half-maximal activation and inactivation ($V_{1/2}$) were 3.6±1.6 and -18.9±1.1 mV, with the curves having Boltzmann slopes (k) of 15.7±0.6 mV and 9..9±0.7 mV, respectively. Averaged maximal Ca^{2+} conductance of 1.34±0.23 nS and very positive reversal potential of the current, 55.5±2.9 mV, suggested adequate L-I_{Ca} isolation.

The 'window current', which is maximal at the potential of intersection of these curves, indicates clearly that a persistent I_{Ca} is present over a wide range of potentials including within the physiological range of resting potentials in this cell-type and those achieved during PE action, with peak at about -10 mV (inset in Fig.1B).

After examining the activation/inactivation characteristics of the persistent L-I_{Ca}, a different protocol was used to investigate the direct effects of TRPM8 agonists on L-I_{Ca}. Menthol has been shown previously to inhibit L-I_{Ca} in other cell types, namely neurons and cardiac myocytes (6, 36), suggesting, based on molecular similarities of channels, the presence of such inhibition in vascular myocytes as well. In the present study, using the same menthol concentration (300 µM) as used in our previous tensiometric experiments (17), we observed strong inhibitory effects of menthol on L-I_{Ca} in tail artery VSMCs (Fig. 1C). Following subsequent nifedipine application for several minutes, a residual transient current was often observed. This current likely represented Ca^{2+} influx through T-type VGCCs which are co-expressed with L-type channels in a sub-population of rat tail artery VSMCs (28). Inactivation kinetics of L-I_{Ca} could be well approximated by the sum of two exponentials, and in the presence of menthol it was clearly accelerated (e.g. in Fig 1C τ_{fast} was reduced by menthol from 26.6 to 21.0 ms). Thus, menthol is likely to be a gating modifier, rather than a channel blocker that simply hinders the channel pore.

A time-course plot showing mean peak and late L-I_{Ca} for eight cells tested (C_m=33.1±1.8 pF; n=8; N=3), under control conditions and in the presence of vehicle-only or menthol application, is illustrated in Figure 1D. Menthol was found to

significantly reduce peak L-I_{Ca} by 32.9±2.7 % compared to a 7.6±1.6 % reduction induced by the vehicle only control (0.3 % ethanol; *P*<0.001; n=8; N=3), while the late L-I_{Ca} was significantly reduced by 67.0±7.0 % compared to a 18.8±5.5 % reduction induced by the vehicle only control (*P*<0.001; n=8; N=3). These findings are summarised in Fig. 1E.

These findings strongly suggest that the vasodilatory effects of menthol on pre-constricted vascular rings, as documented previously by Johnson *et al.* (17) and Cheang *et al.* (10), may largely be accounted for by a direct inhibitory action on VGCCs opened by either membrane depolarisation induced by KCl or phenylephrine (PE) (hypothesis 1). Fig. 1F shows that, indeed, a substantial part of PE-induced contraction is underlined by VGCC activity.

However, if a major indirect effect of menthol (e.g. non-TRPM8 related) on vascular tone is vasorelaxation via the inhibition of VGCCs, it is likely that TRPM8 channels present in vessel are also activated but that their role in vascular tone is masked by the more robust action of L-I_{Ca} inhibition. Therefore, to minimise this secondary effect ,one method would be to apply menthol to unstimulated VSM, when VGCCs are largely closed at resting potential and L-I_{Ca} contribution is minimal (see "window" current-voltage relation in Fig 1B, inset). As such, the application of activators of a Ca^{2+}-permeable ion channel in VSM would be expected to induce an increase in $[Ca^{2+}]_i$, according to Yang *et al*. (42), and vasoconstriction (hypothesis 2). Indeed, such vasoconstrictions have been observed previously when menthol was applied to pre-tensed rat tail artery sections under resting conditions (17).

Effect of TRPM8 agonists on VSM $[Ca^{2+}]_i$ and basal vascular tone

In rat tail artery VSMCs, menthol induced elevations in $[Ca^{2+}]_i$ as well as causing concentration-dependent vasoconstrictions in tail artery vascular rings (Figs. 2 and 3). Enzymatically isolated VSMCs, loaded with fura-2-AM responded to menthol (300 µM) application with robust and reproducible F_{340}/F_{380} ratio increases, responses that were comparable in size to the responses induced by the application of sub/supra-maximal concentrations of the ryanodine receptor (RyR) channel activator caffeine (1-10 mM) and α_1-adrenoceptor agonist PE (2-10 µM) when tested in the same cell (Fig. 2A, B). The summary bar chart shows the peak increases in F_{340}/F_{380} (mean ± S.E.M.) induced by caffeine (1 mM: P<0.05, Student's paired t-test,

n=5, N=4; 10 mM: P<0.001, n=14, N=5), PE (2 & 10 μM) and menthol (300 μM), relative to basal F_{340}/F_{380} prior to each compound application (Fig. 2C).

However, there was one notable difference in the rate of rise of these various Ca^{2+} signals (quantified as time from 10 to 90% of its maximal size, or t_{10-90}), as menthol-induced responses were significantly slower compared to caffeine- or PE-induced responses (Fig. 1D, E). This observation suggests that the density of TRPM-mediated Ca^{2+} influx into the cytosol should be considerably lower compared to the pathways that involve α1-adrenoceptor and ryanodine receptor activation.Consistent with these findings at the cellular level, application of incremental concentrations of menthol to pre-tensed, non-constricted tail artery vascular rings at room temperature (20-22^{O}C) induced vasoconstrictions that were concentration-dependent (100 μM: 0.020±0.003 g or 4±1% of KCl vasoconstriction; n=29, N=14; 300 μM: 0.062±0.010 g or 10±1 % of KCl; n=29, N=14; NS ; 500 μM: 0.115±0.013 g or 17±2 % of KCl; n=29, N=14; 1 mM: 0.339±0.032 g or 52±5 % of KCl; n=29, N=14) and were significantly greater than baseline (300 μM – *P*<0.01; 500 μM – *P*<0.001; 1 mM – *P*<0.001; peak amplitudes significantly different from zero, one-way ANOVA; Fig. 3). When menthol (300 μM) was applied to pre-tensed, relaxed tail artery vascular rings at 37°C vasoconstrictions were significantly smaller (0.025±0.004 g or 3±1 % of KCl; n=8, N=3; *P*<0.01; unpaired *t*-test with Welch's correction) than those observed at room temperature and were more transient in appearance (see Fig. 3A, *inset*). These results are consistent with synergy between cold and menthol in TRPM8 activation, e.g. menthol was shown to activate TRPM8 much more efficiently at 22^{O}C compared to 35^{O}C (27). Clearly, menthol mediates Ca^{2+}-mobilising effects sufficient to trigger significant vasoconstrictions.

Effect of TRPM8 agonists on vascular tone independent of VGCC activity

Consistent with the above results, menthol had an initial rapid vasoconstrictive effect of variable amplitude when applied during PE-induced vasoconstrictions under control conditions (compare Fig. 4A, left panel, Fig. 4B and Fig. 11A, control trace, average menthol peak amplitude was 108.4±7.3 % of PE control, P<0.01, one-way ANOVA, n=26, N=19), but caused a significant slower vasodilatory effect (Fig. 4A,B). The steady-state inhibition of contraction was observed after 10 minutes exposure (menthol late) amounted to 73.5±2.4 % of PE control (P<0.001), as illustrated in Fig.

4A, left panel and Fig. 4B. We reasoned that if the above described VGCC inhibition by menthol (Fig. 1) was indeed involved in the vasorelaxation, prior inhibition of VGCC activity by a saturating nifedipine dose (10 µM) should reveal the specific TRPM8-mediated component in vascular response. Application of nifedipine 10 minutes prior to PE resulted in a 46.5±3.0 % reduction of the PE vasoconstriction amplitude (Fig. 1F; n=41, N=15), while the application of menthol during nifedipine-reduced PE vasoconstrictions resulted in significant additional constriction, presumably mediated by TRPM8 activation (Fig. 4A, right panel). Under these conditions, menthol application induced a peak increase in tone to 163.1±6.0 % of the nifedipine-reduced PE control (P<0.001, n=22, N=13) while the tension remaining 10 minutes after menthol application (menthol late) was also significantly elevated (to 138.4±5.6 %, P<0.001). When the normalised responses to menthol application in control conditions and in the presence of nifedipine were compared, it was found that peak and late menthol (% PE control) responses were significantly greater in the presence of nifedipine (peak: P<0.001, Student's paired t-test; late: P<0.001) (Fig. 4B, right panel).

These findings indicate that the most likely mechanism of menthol-induced vasodilatation of pre-constricted vessels ((17) and Fig. 4A, left panel) was a direct effect on VSM VGCCs, since this effect was reversed in the absence of L-type VGCC contribution to PE-vasoconstrictions (Fig. 4A, right panel). Importantly, these experiments indicate that the inhibition by menthol of L-I_{Ca}, which is more pronounced for its late component (Fig. 1E), also likely masks a substantial part of the vasoconstrictive effects of the compound even in non-stimulated tissue since even at the resting membrane potential considerable "window" Ca^{2+} current (up to 40% of maximum) flows (*inset,* Fig. 1B) contributing to resting vascular tone.

Ca^{2+} store contribution to menthol-induced vasoconstrictions

In different cell types, TRPM8 channels can be expressed not only in the plasma membrane, where they mediate Ca^{2+} influx, but also in the endoplasmic reticulum, where they promote Ca^{2+} release from the intracellular stores (1, 7, 8, 32, 36, 43). In this context, it should be noted that menthol is a membrane permeable molecule. In addition, it is possible that TRPM8-mediated Ca^{2+} influx can trigger Ca^{2+}-induced Ca^{2+} release. To determine whether intracellular Ca^{2+} stores contributed to menthol-induced vasoconstrictions, we inhibited re-uptake of Ca^{2+} into

the sarcoplasmic reticulum (SR) using the sarco/endoplasmic reticulum calcium ATPase pump inhibitor cyclopiazonic acid (CPA). When vessels were pre-incubated with CPA (10 µM) for 30 minutes prior to a PE-induced vasoconstriction, application of menthol (300 µM) 10 minutes later did not induce any increase in tone (peak) as was seen in control conditions, but instead, resulted in a more pronounced vasodilatation that was significantly greater than seen in the absence of CPA (12.2±9.0 % relaxation of PE-vasoconstriction in control conditions compared with 60.6±6.8 % relaxation in the presence of CPA; $P<0.01$, Student's paired *t*-test; n=8, N=8; Fig. 5A,B).

To strengthen the theory that menthol activation of TRPM8 could result in Ca^{2+} release in tail artery VSMCs to contribute to vasoconstriction, immunocytochemistry was performed on isolated tail artery myocytes using a specific TRPM8 antibody. A thin, intense immunofluorescence signal on the perimeter of the cell was identified, as well as a pronounced signal within the deeper cytosol (Fig 5C, left panel). These signals could be attributed to TRPM8 presence at plasmalemma and/or sub-plasmalemmal SR in addition to a large intracellular structure surrounding the nucleus, most likely the SR. This hypothesis was confirmed by the parallel labelling of type I $InsP_3$ receptors (Fig. 5C, middle panel) followed by examination of merged images, which revealed such colocalisation of TRPM8 and $InsP_3R$, as evident by yellow color in Fig. 5C, right panel. These results are consistent with the view that TRPM8 can be localised both on the plasma membrane and/or the SR membrane, and mediates Ca^{2+} influx and/or Ca^{2+} release correspondingly, as in other cell types (1, 7, 8, 32, 35, 37, 44).

This enhanced vasorelaxation after CPA treatment could, at least in part, be attributed a relatively larger contribution of VGCC activity to PE-induced vasoconstrictions when SR was dysfunctional. To distinguish between these possibilities we next focused on the role of SR in menthol-evoked Ca^{2+} responses. In order to evaluate the relative contribution of VGCC-mediated Ca^{2+} influx vs Ca^{2+} store release to menthol-evoked intracellular Ca^{2+} rise, ratiometric Fura-2 measurements were performed on single myocytes, while VGCC were inhibited by nicardipine (5 µM) or Ca^{2+} store was depleted with CPA (10 µM), respectively. While nicardipine had no statistically significant effect, the Ca^{2+} transients in response to menthol were inhibited by 48.9±1.2% (n=3) after Ca^{2+} store depletion (Fig. 6). It should be noted, that this effect of Ca^{2+} store depletion on the amplitude of menthol-

induced Ca^{2+} response is even underestimated since the measured F_{340}/F_{380} signal includes, in addition to the menthol-induced signal, also a more sustained store-operated Ca^{2+} entry component, as can be seen in panel Fig. 6C.

Consistent with this, in confocal Ca^{2+} imaging experiments performed on single Fluo-3 AM loaded myocytes with the use of the MR-A1 system we observed significant intracellular Ca^{2+} rises both with and without external Ca^{2+}, with indications of a propagating through the cytosol Ca^{2+} wave – phenomenon typically attributed to Ca^{2+}-induced Ca^{2+} release process (Fig. 7A-C). On average, in Ca^{2+}-free PSS the F/F_0 signal was reduced from 1.92±0.10 to 1.39±0.03 (n=4; P=0.002) indicating that menthol-induced $[Ca^{2+}]_i$ rises were due to both Ca^{2+} entry and Ca^{2+} release. Moreover, Ca^{2+} responses to subsequent caffeine application were relatively smaller if caffeine was applied in the presence of menthol (F/F_0 2.14±0.16 vs 1.99±0.19 for caffeine and menthol, respectively, n=4; P=0.56), than after menthol wash-out (F/F_0 2.12±0.26 vs 1.39±0.03 for caffeine and menthol, respectively, n=4; P=0.029) (compare Fig. 7A and B). This strongly indicates that menthol, by presumably acting on TRPM8 channels expressed on the SE membrane (Fig. 5C), reduces the Ca^{2+} content that be liberated by caffeine.

To investigate the spatial dynamics of menthol-induced $[Ca^{2+}]_i$ rises at a higher temporal resolution, we employed the spinning Nipkow disc system. With the improved temporal resolution we observed that, indeed, menthol clearly induced a propagating through the cytosol Ca^{2+} wave in the presence (Fig. 7D, top panel) and the absence (Bottom panel) of external Ca^{2+} (Figure 7, bottom panel). Removal of external Ca^{2+} caused a decrease in both in the amplitude and the velocity of propagation of menthol-induced Ca^{2+} wave, as can be seen by the increased time lag between the Fluo-4 signal measured at the initiation (bottom region of cell) and destination site (top region) of the myocyte (Fig. 7E).

Effect of TRPM8 antagonist (AMTB) on menthol-induced vasoconstrictions in the presence of nifedipine

We next examined the selectivity of menthol action with regard to TRPM8 activation, expecting loss of its effect following TRPM8 blockade by AMTB. In the presence of AMTB (10 μM), menthol-induced vasoconstrictions in the absence of VGCC contribution (Fig. 8A, *left panel*) were greatly inhibited (*right panel*). On average, in the presence of nifedipine, menthol-induced peak and late

vasoconstriction amplitudes that were 173.3±10.3 % and 145.9±9.0 % of the PE amplitude, respectively, became significantly reduced to 135.1±6.4 % and 79.5±8.7 % of the PE control, respectively (peak: P<0.01; late: P<0.001; one-way ANOVA; n=9, N=9) when preparations were pre-incubated in nifedipine plus AMTB (Fig. 8B).

Effects of other TRPM8 agonists on I_{Ca} in rat tail artery VSMCs

In addition to menthol, we examined the effects of two other, more recently introduced TRPM8 agonists (icilin and WS-12) on L-I_{Ca} in tail artery VSMCs (Fig. 9). The concentration of icilin tested was the same as used in our previous tensiometric experiments (17) and as deemed sufficient for supramaximal activation of TRPM8 (50 µM). WS-12 was used at its supramaximal concentration of 50 µM (33). As shown in Fig. 9A, both agonists had strong inhibitory effects on L-I_{Ca} in these cells. Similarly to menthol, iclin accelerate current inactivation of L-I_{Ca} (e.g. in Fig 9A τ_{fast} was reduced by icilin from 32.4 to 25.5 ms). Thus, icilin is also likely to be a VGCC gating modifier.

A time-course showing the effects of vehicle-only and icilin application on peak and late L-I_{Ca} is illustrated in Fig. 9B. Icilin significantly reduced peak I_{Ca} by 35.8±2.6 %, compared with a 11.0±1.8 % reduction induced by the vehicle-only control (0.5 % DMSO; P<0.001; n=8; N=3), and caused a 78.5±5.7 % inhibition of late I_{Ca} compared with 17.2±3.0 % by its vehicle-only (P<0.001; Fig 9C). WS-12 induced a 30.0±2.2 % reduction in peak L-I_{Ca} compared with 16.3±1.7 % by its vehicle-only control (0.5 % ethanol; P<0.001), but there was no significant difference between the effects of drug and vehicle-only control on late L-I_{Ca} (55.3±4.8 % reduction vs. 40.5±4.1 %; Fig. 9D). These observations in vascular cells contrast to those in rabbit cardiac myocytes where icilin appeared to have no effect on L-I_{Ca} (6).

The latter observation that WS-12 had little or no effect on the sustained L-I_{Ca} suggested that this menthol derivative is a more selective TRPM8 agonist compared to menthol. We have thus performed similar to the above described Ca^{2+} ratiometric measurements using Fura-2 AM loaded myocytes (Fig. 10) and contractile recordings (Fig. 10B, data summarised in Fig. 10C). WS-12 (10 µM) induced robust $[Ca^{2+}]_i$ rises comparable to those induced by caffeine, PE or menthol (Fig. 10A). However, similar to menthol, the rise phase of these responses was much slower compared to caffeine- and PE-induced responses, with $t_{10\text{-}90}$=6.16±1.97 s (n=4) (compare to Fig. 2E). When applied in the presence of PE WS-12 invariably induced

an additional contraction, which was augmented in the presence of nifedipine (Fig. 10B, C). This indicates, that although some L-I_{Ca} occurred (i.e. vehicle only effect in case of late L-I_{Ca}), the associated relaxation component was masked by a more pronounced contractile component due to TRPM8 activation.

Discussion

The present study confirms that vasodilatory effects of menthol on pre-constricted vascular rings (10, 17) are mediated via a direct inhibitory action on L-type VGCCs. We thus refer to this effect as nonspecific if menthol is employed as pharmacological tool for the investigation of physiological effects of TRPM8 activation. When organ bath tensiometric experiments were performed on tail artery rings in the presence of nifedipine, TRPM8 agonist-induced vasodilatations were abolished indicating that these were a consequence of a direct inhibition of L-type VGCC activity. However, it should be noted that moderate contribution of endothelium to these relaxations was observed (17), and this likely holds true considering VGCC are lacking in endothelial cells, although further tests of endothelium involvement are now needed using experimental protocols we developed here for testing TRPM8 specific roles. However, in the present study the endothelium was removed to avoid this additional complexity.

It is now well documented that menthol interacts with proteins other than TRPM8. It has been shown that the compound can potently inhibit skeletal muscle and neuronal voltage-operated Na^+ channels (15), neuronal and cardiac VGCCs (6, 36) and now smooth muscle VSMCs ((10) and present study). These findings could be expected considering the similarity of vascular to cardiac muscle VGCC molecular composition (9). In addition, menthol has also been shown to activate TRPV3 at mM concentrations and bi-modally activate and inhibit TRPA1 in a concentration-dependent manner (18, 21). Millimolar concentrations have also been shown to induce Ca^{2+} release from the internal stores in non-TRPM8-expressing HEK293 cells (22). Thus, in the present study a combination of a number of different TRPM8 agonists and selective TRPM8 antagonist AMTB were used to determine whether a specific and physiologically relevant TRPM8-mediated effect existed in tail artery VSMCs.

In spite of its robust inhibitory effects on L-I_{Ca}, we show that menthol induces robust Ca^{2+}-mobilising effects in tail artery VSMCs that were comparable with caffeine and PE. In addition, at room temperature, menthol induced concentration-dependent vasoconstrictions in the organ bath preparation. Cheang *et al*. (10) noted that they did not observe vasoconstrictive effects of menthol in rat aorta, mesenteric artery or coronary artery at 37°C. This is in agreement with our own observations (unpublished). Menthol vasoconstrictions were only investigated in tail artery

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vascular rings in the present study, and moreover, were larger and observed more consistently at room temperature. Clearly, the very complex interactions between voltage, menthol and temperature in TRPM8 activation (2, 14, 40) may explain these differences depending on temperature at which experiments are performed, as there is strong synergy between cooling and menthol (2, 15, 43). Indeed, according to Peier *et al.* (27) study, menthol was rather inefficient activator of TRPM8 at 35^{O}C compared to 22^{O}C. Interestingly, when experiments were performed in the presence of nifedipine, we were able to observe prominent menthol-induced vasoconstrictions in PE preconstricted arteries obviously masked by the inhibitory action of menthol on L-type Ca^{2+} currents described in previous studies (10, 17). In addition, menthol application to the organ bath while vascular rings were at basal tone also produced significant vasoconstrictions, though these were usually only observed when experiments were performed at room temperature. The observation that menthol-induced vasoconstrictions became more pronounced at 37^{o}C in the presence of PE (compare Fig. 8A and Fig. 3A) could be explained by several factors. First, we found significant colocalisation between TRPM8 and $InsP_3R1$ (Fig. 5C). Since $InsP_3R$ activity is potentiated by $[Ca^{2+}]_i$ rise it is possible that Ca^{2+} efflux via TRPM8 in its close vicinity potentiates PE-induced $Ca2^{+}$ signalling via $InsP_3R$. Second, TRPM8 has been recently shown to activate G_q proteins directly, and thus it can synergise with α1-adrenoceptor in this same action (20). Third, although Ca^{2+} influx via TRPM8 makes prominent contribution to the menthol-induced Ca^{2+} responses (Fig. 7A,B,E), one should not overlook the importance of Na^{+} influx, as TRPM8 is a nonselective cation channel primarily admitting Na^{+} under physiological gradients. Indeed, Na^{+} entry via the Na^{+}-Ca^{2+} exchanger (NCX) facilitates Ca^{2+} entry via the NCX operating in the reverse mode, and this way it contributes to PE-induced $[Ca^{2+}]_i$ oscillations and vasoconstriction. This mechanism becomes even more important in the presence of nifedipine (21), which is consistent with our observations assuming that TRPM-mediated Na^{+} entry facilitates Ca^{2+} entry via the reverse-mode of NCX.Mahieu *et al*. (22) described a TRPM8-independent menthol- mediated Ca^{2+} release observed in four different non-TRPM8 expressing cell-lines. In contrast to our observations however, the menthol responses observed by Mahieu *et al*., were temperature-dependent with large responses measured at 33°C, but only minor responses at room temperature, the opposite to our observations in rat tail artery smooth muscle. If the Ca^{2+}-mobilising and vasocontrictive effects of menthol in the

present study were indeed a non-specific effect of the compound, it is reasonable to suggest that they would induce similar non-specific effects in other vascular beds. Menthol was applied to organ bath preparations of rat aorta, mesenteric artery and femoral artery vascular rings at both room temperature and 37°C, but had no effect on basal tone (data not shown).

We found that menthol-induced vasoconstrictions in the presence of PE were mediated, at least in part, by Ca^{2+}-release from the internal stores, since they were abolished by CPA pre-treatment which is known to deplete the Ca^{2+} store. Moreover, in the absence of the vasoconstrictive component of menthol's action after Ca^{2+} store depletion, the vasodilatory effect of the compound was significantly greater. The involvement of TRPM8 in Ca^{2+} release is further supported by the localization of anti-TRPM8 immunofluorescence signals in close proximity to type I $InsP_3$ receptors on intracellular structures analogous to SR (41) in immunocytochemistry experiments performed on isolated rat tail artery VSMCs. In mouse mesenteric artery myocytes, TRPM8 is also mainly localised in the cytosolic region (35). In menthol induced $[Ca^{2+}]_i$ transients, the large initial transient component was also suggestive of predominant Ca^{2+} release contribution.

Furthermore, a significant population of these intracellular TRPM8 channels showed colocalisation with type I $InsP_3$ receptors, although it is possible that another part of intracellular TRPM8 staining simply represents synthesis and transport of TRPM8 proteins. Despite this uncertainty, in our Ca^{2+} confocal imaging experiments we obtained strong evidence for the contribution of both Ca^{2+} influx and Ca^{2+} release (the latter was evident after external Ca^{2+} removal) to $[Ca^{2+}]_i$ rises induced by menthol, and we also found that menthol partially reduces the caffeine-releasable Ca^{2+} pool. In addition, ratiometric Ca^{2+} measurements showed significant reduction of menthol-induced Ca^{2+} transients following Ca^{2+} store depletion by CPA treatment. Taking together, these observations provide strong structural and functional evidence for the role of TRPM8 channels expressed in the SR. Notably, however, menthol (and WS-12) $[Ca^{2+}]_i$ rises developed much more slowly compared to those initiated by PE (mainly via activation of $InsP_3$ receptors) and caffeine (via activation of RyR receptors). This difference can in part be explained by slower diffusion of TRPM8 agonists into the cytosol, but it is more likely that lower expression of TRPM8 channels and/or the lack of their positive feedback activation by Ca^{2+}, as is

the case for both $InsP_3$ and RyR receptors, can account for such differences in kinetics.

Furthermore, menthol-induced vasoconstrictions were significantly reduced in the presence of the selective TRPM8 antagonist AMTB, as an additional independent test of specificity. Notably, the physiologically-relevant involvement of TRPM8 in Ca^{2+} release mediated by the intracellular (ER) TRPM8 channels has also been documented in other cells types, such as DRG neurons, normal prostate secretory epithelial cells, and in LNCaP cells, a prostate cancer cell-line (7, 8, 37, 39, 44).

While menthol has previously been shown to inhibit VGCCs, the present study is the first that we know of that describes inhibitory effects of icilin and WS-12 on L-I_{Ca}. Baylie *et al*. (6) did not observe any inhibitory effect of icilin on L-I_{Ca} at concentrations up to 100 µM in rabbit cardiac myocytes. The reason for this difference in L-VGCC inhibition by icilin may be any combination of, differences between the experimental conditions employed and species (e.g. rabbit vs. rat) or tissues studied. In the present study, electrophysiological experiments were performed at room temperature, while Baylie *et al*. recorded the effects of icilin on L-I_{Ca} at 35°C. In addition to potential species and tissue-specific (vascular vs. cardiac) differences in L-VGCC subunit composition (e.g. in their α2δ- and β-subunits) and sequence variations, there is further possibility for variation of responses to pharmacological reagents due to tissue-specific alternative splicing resulting in altered channel structures (11, 19). Among the three TRPM8 agonists, WS-12 appears to be the most selective one, at least it did not affect late component of L-I_{Ca} (although this was still inhibited to some extent by vehicle). Correspondingly, the vasorelaxation phase was absent when WS-12 was applied in the presence of PE.

In conclusion, the TRPM8 agonist menthol has dual effects on vascular tone, normally resulting in a biphasic response (trace 1 in Fig. 11A). Menthol causes an endothelium-independent non-specific vasodilatory effect as a result of L-I_{Ca} inhibition, which could be “unmasked” by interrupting normal Ca^{2+} release (trace 2 in Fig. 11A), and a TRPM8-mediated vasoconstriction effect that was at least partly dependent on normal Ca^{2+} release, which could be “unmasked” under conditions in which VGCC activity was minimised (trace 3 in Fig. 11A). We thus propose that TRPM8 is expressed and functional in rat tail artery VSM and that it is potentially localised both on the plasma membrane and the sarcoplasmic reticulum, the latter

predominantly functionally relevant, as illustrated in our summary schematic model (Fig. 11B). Sun et al. (35) have recently provided evidence for beneficial effects of TRPM8 activation by menthol in hypertension treatment. Although much remains to be learned about the pathophysiology of TRPM8 activation by cold, environmental cold is a well-known risk factor for hypertension. Other conditions where the present findings may have translational implications include cardiovascular surgery, which is often performed at temperatures below 37^{O} C, where TRPM8 can be activated, organ storage and transplantation, and Raynaud's phenomenon.

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Conflicts of interest

None.

Figure legends

Figure 1

Investigation of L-I_{Ca} in rat tail artery VSMCs indicates the existence of non-inactivating "window" current in these cells and L-I_{Ca} inhibition by TRPM8 agonist, menthol.

A - Representative recording of L-I_{Ca} in a rat tail artery VSMC, elicited by stepping the membrane potential to a range of prepulses (-100 to +40 mV with a 10 mV increment; 500 ms duration) from a holding potential of -60 mV to determine channel steady-state activation properties before stepping to +10 mV for 50 ms in order to determine the steady-state inactivation. The upper panel shows the voltage protocol.

B - Plotting activation and inactivation curves for the L-I_{Ca} demonstrated an overlap region whereby a small but persistent current exists, known as a "window" current. Inset: data from 8 cells (C_m=42.2±4.0 pF; n=8; N=4) were used to calculate mean "window" currents in tail artery VSMCs.

C - Representative current traces showing the effects of menthol (300 µM) and the specific L-type VGCC blocker nifedipine (10 µM) on *L-I_{Ca}* in the same rat tail artery VSMC. Currents were evoked by step depolarisation from -60 mV to +10 mV for 400 ms, applied every 10 seconds.

D - Example time-course showing the inhibitory effects of vehicle-only (0.3% ethanol) and menthol (300 µM) application on mean peak and late L-I_{Ca} in rat tail artery VSMCs (33.1±1.8 pF; n=8; N=3).

E - Peak and late L-I_{Ca} were compared in individual cells in control conditions and in the presence of vehicle only (V; 0.3% ethanol) or menthol (M; 300 µM). Both peak and late L-I_{Ca} in the presence of menthol were significantly reduced compared with their vehicle-only condition ($P<0.001$; n=8; N=3).

F - Representative organ bath tensiometric trace showing that nifedipine, applied at the same concentration (10 µM) strongly suppresses the sustained component of the phenylephrine (2 µM)- evoked contraction, which is most likely mediated by the "window" L-I_{Ca} in rat tail artery VSMCs.

Figure 2

TRPM8 agonists induced intracellular Ca^{2+} responses in rat tail artery VSMCs.
A - Ratiometric Ca^{2+} recording of representative responses to caffeine (10 mM), PE (10 µM), and menthol (300 µM) with increases in F_{340}/F_{380} ratio in a rat tail artery VSMC.
B - Ratiometric Ca^{2+} recording of a representative rat tail artery VSMC responding to repeated applications of menthol (300 µM), followed by the application of caffeine (1 mM), with increases in F_{340}/F_{380} ratio.
C - Summary data of the peak increases in F_{340}/F_{380} in the presence of caffeine (1 and 10 mM), phenylephrine (2 and 10 µM) and menthol (300 µM) relative to baseline ratio prior to applications. Student's paired *t*-test was used to compare peak increases in ratio for each compound to mean baseline in the 30-60 seconds prior to application.
D – Intracellular calcium responses recorded in the same myocytes were normalised to baseline and superimposed on the same time-scale to illustrate the differences in their 1-to90% rise-time, as illustrated for the menthol trace.
C - Summary data of mean rise-times of $[Ca^{2+}]_i$ responses to caffeine (10 mM, n=11), PE (10 µM, n=4) and menthol (300 µM, n=11). The Kruskal-Wallis non-parametric ANOVA with Dunn‟s post hoc test was used to compare these values.

Figure 3
Menthol induces concentration-dependent vasoconstrictions in tensiometric recordings of tail artery vascular rings.
A - Representative organ bath tensiometric traces showing concentration-dependent vasoconstrictions induced by menthol applications to rat tail artery vascular rings at room temperature. *Inset,* menthol-induced vasoconstrictions observed at 37°C were smaller in amplitude and more transient in appearance than those observed at room temperature.
B – Bar chart summarising the peak amplitudes of menthol-induced vasoconstrictions relative to 60 mM KCl-induced vasoconstriction. Menthol concentrations 0.3-1 mM were sufficient to induce vasoconstrictions that were consistently and significantly greater than baseline tone (one-way ANOVA, n=29; N=14).

Figure 4

In the absence of VGCC activity, menthol induces vasoconstrictions in rat tail artery vascular rings.

A - Representative tensiometric trace showing the predominant vasodilatory effect of menthol (300 µM) on PE-constricted rat tail artery vascular rings, which was suppressed while the constrictive effect was “unmasked” in the presence of the L-type VGCC blocker, nifedipine (10 µM; n=22, N=13).

B - Bar chart summarising the effects of menthol on PE-vasoconstriction, causing an additional vasoconstriction (denoted Peak) followed by a later vasodilatory effect measured 10 minutes after menthol application (denoted Late), under control conditions and when nifedipine was applied 10 minutes prior to the application of PE. Summary data have been normalized to the PE-vasoconstriction amplitude 10 minutes after PE application, as illustrated in the inset, namely: Peak = B/A; Late = C/A and expressed as % of PE-response before menthol application. Values for each condition, i.e. PE after 10 minutes, menthol peak and after 10 minutes (Late) were then compared by one-way ANOVA for repeated measures followed by Tukey’s post hoc test. Peak and late values in control conditions and in the presence of nifedipine were compared by Students t-test

Figure 5

Store-depletion in rat tail artery vascular rings abolished menthol-induced vasoconstrictions and potentiated menthol induced vasodilatations indicating TRPM8 predominant functional localisation on the VSM SR, which was examined by immunocytochemistry.

A - Representative trace showing the vasodilatory effect of menthol (300 µM) on PE (2 µM) vasoconstrictions of rat tail artery vascular rings (left panel, control) is amplified when CPA (10 µM) is applied 30 minutes prior to PE (n=8, N=8).

B - Summary data showing that menthol-induced vasodilatations in the presence of CPA were significantly greater than in control conditions as determined by comparing the PE amplitude remaining 10 minutes after menthol application in both conditions (P<0.01, Student’s paired t-test; n=8, N=8).

C - Representative cell (n=5, N=2) was fixed, blocked and permeabilised, and then incubated in Alomone anti-TRPM8 antibody (1:200) followed by Alexa 488

secondary antibody (1:200) and mounted in DAPI-containing medium. Left panel shows TRPM8 staining only. Middle panel shows a composite of TRPM8 and DAPI staining. Right panel shows a representative permeabilised cell, which was incubated only in the Alexa 488 secondary antibody (white lines = 20 μm).

C – Confocal images of a representative rat tail artery myocyte co-labelled with Alomone anti-TRPM8 antibody (1:200, green channel) and anti-type I $InsP_3$ receptor ($InsP_3R1$) antibody (1:100, red channel) indicate both perimembrane and intracellular subcellular localisation of TRPM8, while the presence of many yellow pixels in the composite image indicates its significant colocalisation with $InsP_3R1$. Fluorescence signals were normalised to maximum values for each channel to allow for differences in fluorescence intensity.

Figure 6

Ratiometric $[Ca^{2+}]_i$ measurements in fura-2 AM loaded rat tail artery myocytes indicate that inhibition of VGCC has no effect, while Ca^{2+} store depletion strongly reduces the amplitude of menthol-induced $[Ca^{2+}]_i$ transients in resting cells.
A – In control, two consecutive menthol (300 μM) applications evoked reproducible $[Ca^{2+}]_i$ rises.
B – In cells treated for 5 min with the selective L-type Ca^{2+} channel blocker nicardipine (5 μM), the Ca^{2+} response to menthol was only marginally reduced.
C - After calcium store depletion induced by CPA (10 μM) treatment for 5 min the menthol–induced Ca^{2+} transient was decreased by about 50%, suggesting that Ca^{2+} release from the SR plays a significant role in the intracellular Ca^{2+} rise due to TRPM8 activation by menthol.
D – Mean normalized to control response F_{340}/F_{380} values summarizing the effects of VGCC inhibition (P=0.065, n=3) and Ca^{2+}-store depletion (P=0.002, n=3) on menthol-evoked $[Ca^{2+}]_i$ transients.

Figure 7

Laser-scanning confocal microscopy of Fluo-3 AM or Fluo4-AM loaded rat tail artery isolated myocytes indicates both Ca^{2+}-influx and release in response to menthol application, as well as menthol-induced Ca^{2+} waves.

A – Time-course of Fluo-3 emitted fluorescence (detected with the MR-A1 system) in the region of interest (ROI, inset frame), while cell was challenged with two menthol (300 μM, denoted M) applications: first in normal-Ca^{2+} physiological salt solution (2 mM; PSS); second in nominally Ca^{2+}-free PSS, followed by caffeine (10 mM) application in the presence of menthol and still in nominally Ca^{2+}-free PSS. Markers a-d indicates the time points of the frame series illustrated in C.

B – in a similar to the illustrated in A experiment, but with caffeine application (10 mM) after menthol (300 μM, denoted M), comparative larger caffeine-induced signal was observed, suggesting that menthol releases Ca^{2+} from the caffeine-sensitive pool, possibly via an additional Ca^{2+}-induced Ca^{2+} release.

C – Frame series show changes in intracellular Ca^{2+} (i.e. color-coded Fluo-3 fluorescence signal intensity) during the different experimental conditions: a - control conditions in normal-Ca^{2+} PSS; b - in the presence of menthol (300 μM) in normal-Ca^{2+} containing PSS; c - in the presence of menthol (300 μM) in nominally Ca^{2+}-free PSS; d – following caffeine (10 mM) application in the presence of menthol (300 μM) in nominally Ca^{2+}-free PSS. Note menthol-induced $[Ca^{2+}]_i$ rises causing strong myocyte contraction.

D – Confocal frame series showing the spatial dynamics of changes in intracellular Ca^{2+} in response to 300 μM menthol application (i.e. color-coded Fluo-4 fluorescence signal intensity) acquired using the spinning Nipkow disc system at a rate of 33 frames per second in the same tail artery myocytes with (top panel) and without (bottom panel) Ca^{2+} in the external solution.

E - Time-course of Fluo-4 emitted fluorescence recorded at site of Ca^{2+} signal initiation (blue line) and at the opposite side of the myocyte (black line) illustrated in panel D.

Figure 8

TRPM8 antagonist AMTB strongly inhibits menthol-induced vasoconstrictions of rat tail artery vascular rings, which were “unmasked” in the presence of the L-type VGCC blocker nifedipine.

A - Representative tensiometric traces showing the effects of menthol (300 μM) on a PE-constricted rat tail artery vascular ring in the presence of nifedipine (10 μM) and

in the presence of both nifedipine (10 µM) and the TRPM8 antagonist AMTB (10 µM).
B - Summary data quantifying the effects of menthol when applied 10 minutes into a PE-vasoconstriction in control conditions, in the presence of nifedipine, and in the presence of nifedipine plus AMTB. Summary data presented were normalized to the PE-vasoconstriction amplitude 10 minutes after PE application for each experimental condition (% control) as was already illustrated in Fig. 4B, left panel. Menthol peak and late (after 10 minutes) % control values in the presence of nifedipine were significantly higher than those and in the presence of nifedipine and AMTB (peak: $P<0.01$; late: $P<0.001$; one-way ANOVA for repeated measures followed by Tukey's post hoc test; n=9, N=9).

Figure 9
Investigation of L-I_{Ca} inhibition by icilin and WS-12 in rat tail artery VSMCs
A - Representative current traces showing the effects of icilin (50 µM) and WS-12 (50 µM) on the L-I_{Ca} in the same rat tail artery VSMC (same cell as in Fig. 1C). Currents were evoked by step depolarisation from -60 mV to +10 mV for 400 ms, applied every 10 s.
B - Example time-course showing the inhibitory effects of vehicle-only (0.5% DMSO) and icilin (50 µM) application on mean peak and late L-I_{Ca} in rat tail artery VSMCs.
C - Peak and late L-I_{Ca} were compared in individual cells in control conditions and in the presence of vehicle-only (0.5% DMSO; V) or icilin (50 µM; I). Both peak and late L-I_{Ca} in the presence of icilin were significantly reduced compared with the vehicle-only condition ($P<0.001$; n=8; N=3).
D - Peak and late L-I_{Ca} were compared in individual cells in control conditions and in the presence of vehicle-only (0.5 % ethanol; V) or WS-12 (50 µM; W). Peak L-I_{Ca} was significantly reduced in the presence of WS-12 compared with the vehicle-only condition ($P<0.001$; n=8; N=3).

Figure 10
The more selective TRPM agonist, menthol-derivative WS12, induces $[Ca^{2+}]_i$ rises in single Fura-2 AM loaded rat tail artery myocytes and, in tensiometric recordings,

alters the balance between vasoconstriction and vasorelaxation towards predominant contraction.

A - Ratiometric $[Ca^{2+}]_i$ recording of a representative Fura-2-loaded VSMC that responded to caffeine (10 mM), PE (10 μM), WS-12 (10 μM) and menthol (300 μM) with increases in F_{340}/F_{380} ratio.

B - WS-12 induces vasoconstrictions in rat tail artery vascular rings both with and without VGCC activity. Representative tensiometric trace showing that WS-12 (50 μM) had a predominantly vasoconstrictive effect (i.e. no net vasorelaxation phase was evident) on PE-constricted rat tail artery vascular rings that was potentiated in the presence of nifedipine (n=12, N=7).

C - The bar chart on the left shows summary data quantifying the effects of WS-12 when applied 10 minutes into a PE-vasoconstriction, causing a significant vasoconstriction (Peak) followed by a prolonged elevated tone measured 10 minutes after WS-12 application (Late, compared to menthol no relaxation was evident). The bar chart on the right shows summary data of similar measurements performed during incubation with nifedipine applied 10 minutes prior to the application of PE. Both Peak and Late responses were increased, suggesting that some “unmasking” of the contractile response in the absence of VGCC activity still takes place.

Figure 11

Normalised superimposed tensiometric traces demonstrating the effects of menthol on vascular tone under different experimental conditions and a schematic model of the probable underlying mechanisms.

A - Demonstrative tensiometric traces showing representative effects of menthol in control conditions (1), and in the absence of either VGCC (2) or SR Ca^{2+} (3) involvement, as indicated. Responses have been normalised to the tension observed 10 minutes after PE (2 μM) application in each experimental condition. In other words, traces were scaled such that at the moment of menthol application all three had the same amplitude, i.e. the level of tension after 10 minutes, just prior to the application of menthol represents 100% PE-induced contraction.

B - Schematic mechanistic model showing the potential pathways by which the TRPM8 agonist menthol induces its multiple effects on vascular tone through the activation of the intracellular (major) and plasma membrane TRPM8 channels to cause vasoconstriction and through the inhibition of VGCC to cause vasorelaxation.